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LncRNA GASL1 inhibits tumor growth of non-small cell lung cancer by inactivating TGF-β pathway

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Abstract. – OBJECTIVE: Growth-Arrest Associated lncRNA 1 (GASL1) is a newly discovered lncRNA that plays a role as tumor suppressor gene in liver cancer, while its involvement in other malignancies is unknown. We aimed to investigate the involvement of GASL1 in non-small cell lung cancer (NSCLC).

PATIENTS AND METHODS: Tumor tissue and adjacent healthy tissues were collected from 98 patients with NSCLC, and blood samples were collected from both NSCLC patients and healthy controls to detect GASL1 expression. All patients were followed up for 5 years and the diagnostic and prognostic values of GASL1 for NSCLC were evaluated by ROC curve analysis and survival curve analysis, respectively. Correlations between serum levels of GASL1 and clinicopathological data of NSCLC patients were analyzed by Chi-square test. GASL1 overexpression and knockdown cancer cell lines were constructed and the effects on transforming growth factor β1 (TGF-β1) expression and cell proliferation were explored by Western blot and CCK-8 assay, respectively.

RESULTS: We found that lncRNA GASL1 expression was significantly downregulated in tumor tissues than in adjacent healthy tissues. Serum level of GASL1 was also lower in cancer patients than in healthy controls. Serum GASL1 is a sensitive diagnostic biomarker for NSCLC, and low expression level of GASL1 indicated short postoperative survival time. Overexpression of GASL1 upregulated, while GASL1 knockdown downregulated TGF-β1 expression. GASL1 overexpression inhibited, and GASL1 knockdown promoted cancer cell proliferation.

CONCLUSIONS: We found that lncRNA GASL1 may inhibit tumor growth of NSCLC by downregulating TGF-β1.

Key Words:
NSCLC, Growth-arrest Associated lncRNA 1 (GASL1), TGF-β1, Cell proliferation.

Introduction

Malignancy in lung is one of the most frequently diagnosed cancers as well as a leading cause of cancer-related death in the world[1]. Non-small-cell lung cancer (NSCLC) as the most common type of lung cancer accounts for more than 85% of all cases[2]. Tobacco consumption is one of the major causes of lung cancer[3]. Besides that, other factors like air pollution also increase the risk of this disease[4]. The incidence of NSCLC is predicted to be significantly increased in near future due to aggregated environmental pollution, especially in developing countries such as China[5]. Most patients with NSCLC are diagnosed at advanced stages and treatment outcomes are usually poor[1]. Therefore, early diagnosis and treatment are the keys for survival of those patients. It has been reported[6] that the development of NSCLC is accompanied by changes in expression pattern of long non-coding RNAs, which is a subgroup of non-coding RNAs composed of more than 200 nucleotides and have critical functions in physiological processes and progression of human diseases[7]. Growth-arrest Associated lncRNA 1 (GASL1) is a newly discovered lncRNA involved in the pathogenesis of liver cancer [8]. Our study systemically investigated the involvement of GASL1 in NSCLC. We found that GASL1 is downregulated in NSCLC and may inhibit cancer cell proliferation by upregulating transforming growth factor β1 (TGF-β1). We provided a new target for diagnosis, prognosis and treatment of NSCLC.

Patients and Methods

Patients

A total of 98 patients with NSCLC were enrolled in Respiration Three Ward of Shanxi Cancer

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Hospital from March 1st, 2011 to July 31st, 2013. Those patients were diagnosed by pathological examinations and were treated for the first time in our hospital. There were 50 males and 48 females, and age ranged from 25 years to 72 years, with a mean age of 47.4 ± 7.7 years. Patients with other lung diseases, other severe diseases endanger patients' lives were excluded. Patients treated in other hospitals and transferred to other hospital during study were not included. At the same time period, a total of 76 healthy volunteers were also included to serve as control group. There were 48 males and 28 females in control group, and age ranged from 27 to 72 years, with a mean age of 48.3 ± 7.2 years. There were no significant differences in age and gender between patient group and control group. All participants were informed with the experimental protocol and signed informed consent. This study was approved by the Ethics Committee of Respiration Three Ward of Shanxi Cancer Hospital.

Specimen Collection

All NSCLC patients were treated with surgical resection and tumor tissues as well as adjacent healthy tissues were collected during surgical operation. Besides that, blood (15 ml) was extracted from elbow vein of both patients and healthy controls. Blood samples were kept at room temperature for 2 h and then centrifuged at 1800 rpm for 15 min at room temperature to collect supernatant. All samples were kept in liquid nitrogen for long term use.

Cell Lines and Cell Culture

A normal human lung tissue cell line WI-38, and two human NSCLC cell lines NCI-H23 and NCI-H299 were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). Cells of all three cell lines were cultured with Eagle's Minimum Essential Medium (ATCC, Manassas, ATCC, VA, USA) supplemented with 10% FBS in an incubator (35ºC, 5% CO_2). FBS was not added in case of drug treatment.

Cell Transfection

Full-length GASL1 cDNA was inserted into pIRSE2-EGFP vector (Clontech, Palo Alto, CA, USA) to establish GASL1 expression vector. Negative control siRNA (CC UAGUUCACCCUUACGGA) and GASL1 siRNA (GCUGCAAGGGAAAUGACAUCGGUUA) were synthesized by Genepharma (Shanghai, China). Lipofectamine 2000 reagent (Cat. No. 11668-019; Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA) was used to transfect 10 nM vector and 50 nM siRNA into 5 x10^5 cells. Negative control siRNA and empty pIRSE2-EGFP vector were used as negative control.

Cell Proliferation Assay

Single cell suspension with a cell density of 4 x10^4 per ml was prepared, and 100 μl of cell suspension containing 4 x10^3 cells were added into each well of 96-well plate. 10 uL of CCK-8 solution (Sigma-Aldrich, St. Louis, MO, USA) were added 24, 48, 72 and 96 later. Cells were cultured for another 4 h, and Fisherbrand™ accuSkan™ GO UV/Vis Microplate Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) was used to measure OD values at 450 nm.

Real-Time Quantitative PCR

Tumor tissues and healthy lung tissues were ground in liquid nitrogen, and TRIzol reagent (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA) was added to extract total RNA. TRIzol reagent was directly mixed with serum and *in vitro* cultured cells to extract total RNA. NanoDrop™ 2000 Spectrophotometers (Thermo Fisher Scientific, Waltham, MA, USA) was used to determine RNA quality. RNA samples with an A260/A280 ratio between 1.8 and 2.0 were subjected to reverse transcription to synthesize cDNA. PCR reaction system was prepared using SYBR® Green Real-Time PCR Master Mixes (Thermo Fisher Scientific, Waltham, MA, USA). Sequences of primers used in PCR reactions were: 5′-CTGAGGCCAAAGTTTCCAAC-3′ (forward) and 5′-CAGCCTGACTTTCCCT CTTCT-3′(reverse) for GASL1; 5'-GACCTCTATGCCAACACAGT-3' (forward) and 5'-AGTACTTGCGCTCAGGAGGA-3' (reverse) for β-actin. PCR reaction conditions were: 95°C for 40 s, followed by 40 cycles of 95°C for 11 s and 60°C for 34 s. Relative expression level of GASL1 was normalized to endogenous control β-actin.

Western-Blot

In vitro cultured cells were mixed with RIPA solution (Thermo Fisher Scientific, Waltham, MA, USA) to extract total RNA. BCA method was used to quantify RNA samples. Then, 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gel electrophoresis was performed using 20 μg of protein per lane. After gel transfer, blocking was performed by incubating polyvinylidene difluoride (PVDF) membranes with 5% skimmed milk at room temperature for 1 h. After that, membranes were incubated with rabbit anti-TGF-β1 antibody (1:2000, ab92486, Abcam, Cambridge, MA, USA) and anti-GAPDH primary antibody (1: 1000, ab8245, Abcam, Cambridge, MA, USA) overnight at 4°C. On the next day, membranes were further incubated with anti-rabbit IgG-HRP secondary antibody (1:1000, MBS435036, MyBioSource) for 1 h at room temperature. After that, ECL (Sigma-Aldrich, St. Louis, MO, USA) was added to develop signals. Finally, membranes were scanned by MYECL™ Imager (Thermo Fisher Scientific, Waltham, MA, USA), and expression level of TGF-β1 was normalized to endogenous control GAPDH using image J V 1.6 software.

Statistical Analysis

SPSS19.0 (SPSS Inc., USA) was used for all statistical analysis. Count data were analyzed by x^2-test. Measurement data were recorded as (x ± s), and comparisons among multiple groups and two groups were performed by one-way analysis of variance followed by LSD test and unpaired *t*-test, respectively. $p<0.05$ indicated a difference with statistically significant.

Expression of lncRNA GASL1 in Tumor Tissues and Adjacent Healthy Tissues of 98 Patients With NSCLC

LncRNA GASL1 expression in tumor tissues and adjacent healthy tissues collected from all 98 patients were detected by qRT-PCR. As shown in Figure 1, about 91% (89/98) of those patients showed downregulated expression of GASL1 in cancer tissues than in adjacent healthy tissues ($p<0.05$). Only 3 patients showed significantly lower expression level of GASL1 in adjacent healthy tissues than in cancer tissues ($p<0.05$), accounting for 3.1%. No significant differences were found in remaining 6 patients ($p>0.05$). Therefore, downregulation of GASL1 is likely involved in the pathogenesis of NSCL.

Expression of lncRNA GASL1 in serum of Healthy Controls and Patients and the Diagnostic and Prognostic Values

Serum levels of GASL1 in patients and healthy controls were measured by qRT-PCR. As shown in Figure 2a, serum levels of GASL1 were significantly higher in patients with NSCLC than in healthy controls ($p<0.05$). ROC curve analysis was performed to evaluate the diagnostic value of serum lncRNA GASL1 for NSCLC. As shown in Figure 2b, the area under the curve was 0.8867, with 95% confidence interval of 0.8373 to 0.9362 ($p<0.0001$). Patients were divided into high expression group ($n=49$) and low expression group (n=49) according to the median serum level of GASL1. Survival curves of those two groups were plotted based on Kaplan-Meier method and compared by log-rank *t*-test. As shown in Figure 2c, survival of patients in high expression group was significantly better than that in low expression group ($p<0.001$). Those data suggest that serum GASL1 may serve as a potential diagnostic and prognostic biomarker for NSCLC.

Correlation Between Serum Levels of lncRNA GASL1 and Clinical Data of Patients with NSCLC

Correlations between serum levels of lncRNA GASL1 (high and low) and clinical data of patients

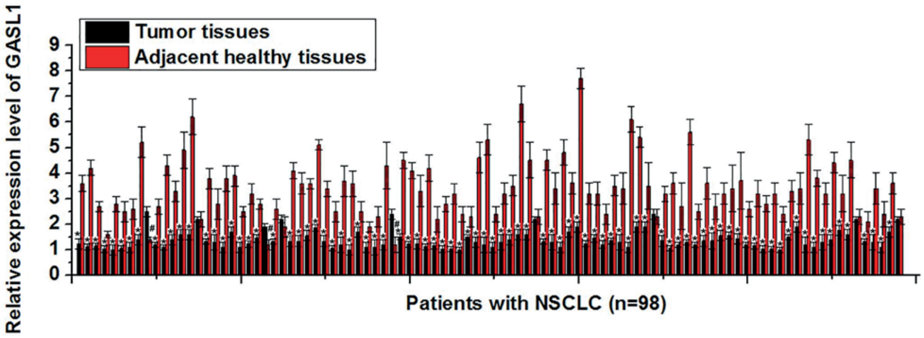

Figure 1. Expression of NSCLC in cancer tissues and adjacent healthy tissues collected from all 98 patients with NSCLC. Notes: *compared with adjacent healthy tissue collected from the same patient, p<0.05; #compared with cancer tissue collected from the same patient, $p<0.05$

Table I. Correlation between serum levels of lncRNA GASL1 and clinical data of patients with NSCLC.

Items	Groups	Cases	High-expression	Low-expression	χ^2	*p*-value
Gender	Male	50	27	23	0.65	0.42
	Female	48	22	26		
Age	>45 (years)	46	20	26	1.47	0.22
	<45 (years)	52	29	23		
Primary tumor diameter	> 7 cm	26	8	18	16.35	0.001
	5 -7 cm	24	7	17		
	3-5 cm	21	15	6		
	< 3 cm	27	19	8		
Distant tumor metastasis	Yes	44	20	24	0.66	0.22
	No	54	29	25		
Smoking	Yes	58	30	28	0.17	0.68
	No	40	19	21		
Drinking	Yes	66	35	31	0.74	0.39
	No	32	14	18		

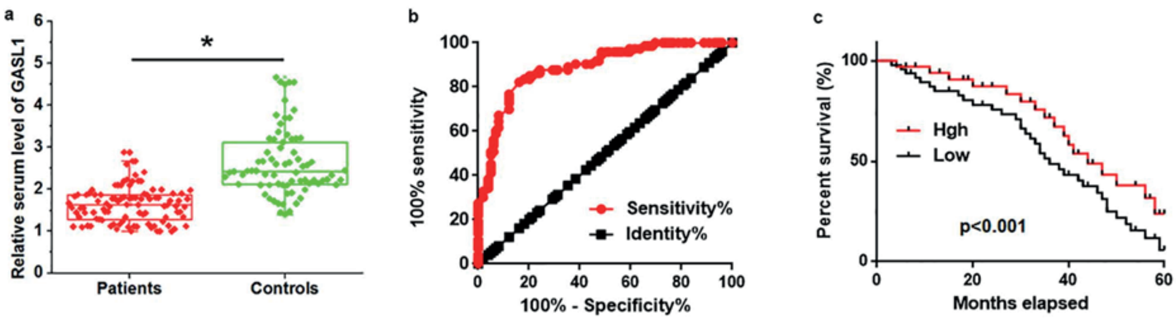

Figure 2. Expression of lncRNA GASL1 in serum of healthy controls and NSCLC patients and the diagnostic and prognostic values for NSCLC. This figure shows the comparison of serum levels of GASL1 between healthy controls and NSCLC patients (a), diagnostic values of serum GASL1 for NSCLC analyzed by ROC curve analysis (b) and comparison of survival curves of patients with high and low serum level of GASL1. Notes: *, $p<0.05$

with NSCLC were analyzed by x^2-test. As shown in Table I, serum levels of lncRNA GASL1 showed no significant correlations with gender, age, distant tumor metastasis, as well as patients' smoking and drinking habits. However, serum level of EPEL was significantly correlated with tumor size.

Effects of lncRNA GASL1 Overexpression and Knockdown on TGF-β1 Expression

Data in Table I indicated that lncRNA GASL1 is likely involved in the growth of NSCLC. It is well known that TGF-β1 inhibits lung cancer cell proliferation [9]. In this study, GASL1 expression vector and siRNA were transfected into the cells of a normal human lung tissue cell line WI-38, and two human NSCLC cell lines NCI-H23 and NCI-H299; the effects on TGF-β1 expression were observed. As showed in Figure 3, GASL1 overexpression upregulated (Figure 3a) and GASL1 knockdown (Figure 3b) downregulated the expression of GASL1 in cells of NSCLC cell lines, but not in cells of the normal lung cell line. Futhermore, TGF-β1 (10 ng/ml) treatment showed no significnat effects on GASL1 expression in all 3 cell lines (data not shown).

LncRNA GASL1 Overexpression Inhibited and GASL1 Knockdown Promoted NSCLC Cell Proliferation

CCK-8 assay was performed to investigate the effects of LncRNA GASL1 overexpression and knockdown on cell proliferation of a normal human lung tissue cell line WI-38, and two human NSCLC cell lines NCI-H23 and NCI-H299. As shown in Figure 4, GASL1 overexpression inhibited and knockdown promoted cell proliferation

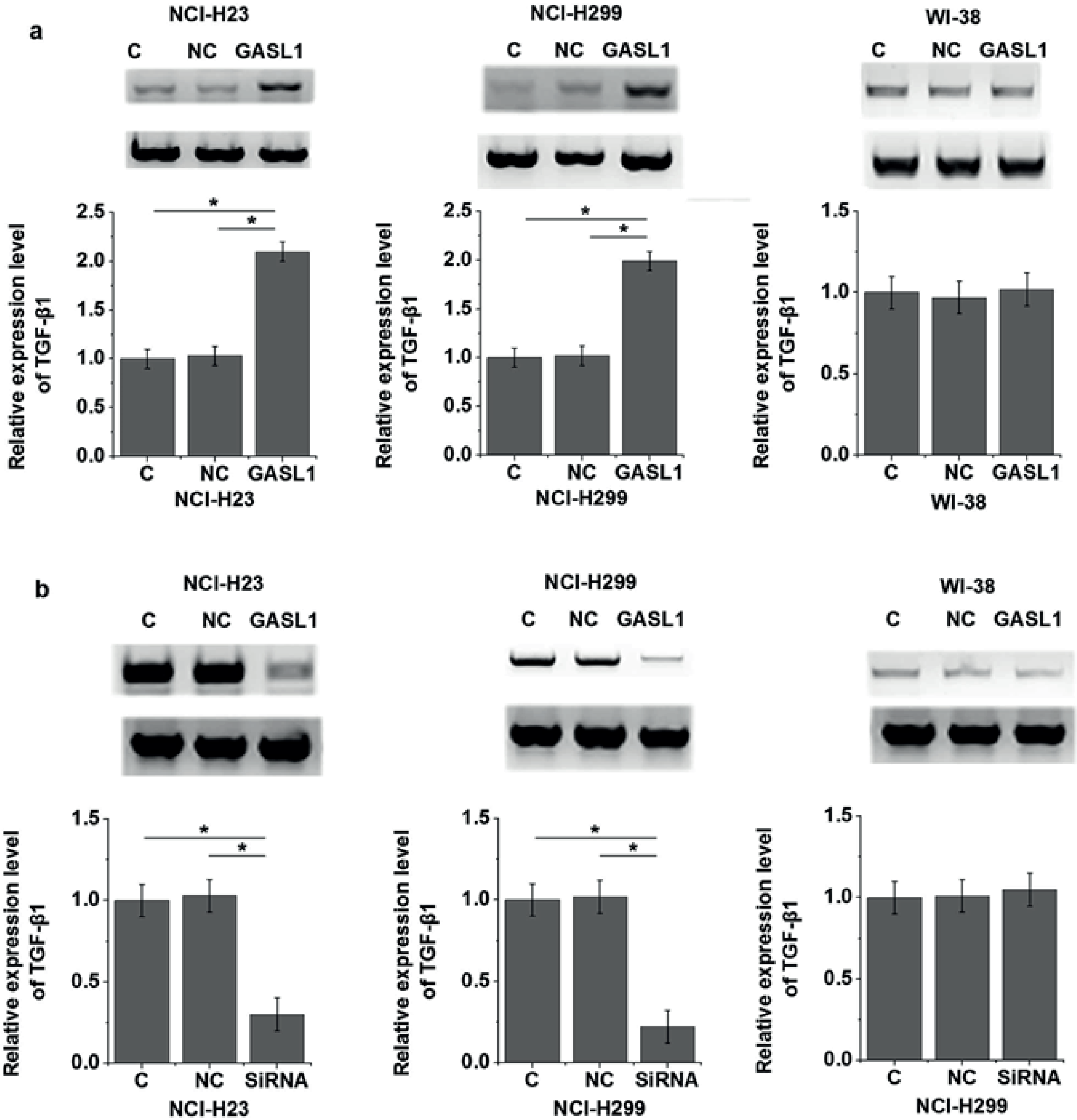

Figure 3. Effects of lncRNA GASL1 overexpression and knockdown on TGF-β1 expression. This figure shows the effects of lncRNA GASL1 overexpression (a) and knockdown (b) on TGF-β1 in different cell lines. Notes: *, $p<0.05$.

of NSCLC cell lines NCI-H23 and NCI-H299, but not normal human lung tissue cell line WI-38. In addition, TGF-β1 (10 ng/ml) treatment reduced the enhancing effects of GASL1 knockdown on NSCLC cell proliferation.

Discussion

Involvement of lncRNAs in the development and progression of NSCLC has been extensively studied in last several decades. LncRNA HOTAIR and SBF2-AS1 are two oncogenic lncRNAs with upregulated expression in NSCLC [10,11]. In contrast, lncRNA MEG3 plays role as tumor suppressor gene and shows downregulated expression pattern in this disease[12]. GASL1 is a newly discovered lncRNA with reduced expression level in liver cancer tissue compared with paired normal liver tissues[8], indicating the role of GASL1 as a tumor suppressor gene in live cancer. In this study, most NSCLC patients showed

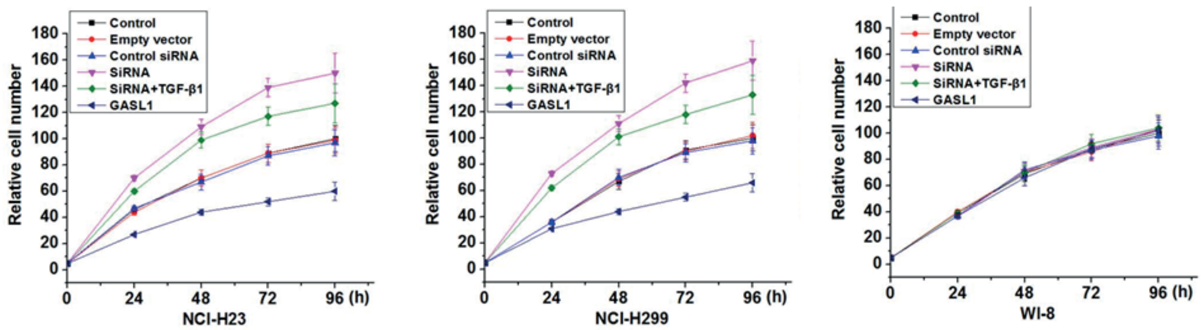

Figure 4. LncRNA GASL1 overexpression inhibited and GASL1 knockdown promoted NSCLC cell proliferation. Notes: *, $p<0.05$.

downregulated expression of GASL1 in cancer tissues than in adjacent healthy tissues, and lower expression level of GASL1 in adjacent healthy tissues than in cancer tissues was only observed in 3 patients. Those data suggest that GASL1 may also play a role as tumor suppressor gene in NSCLC. Treatment of NSCLC is challenged by the existing of distant tumor metastasis in most patients by the time of diagnosis [1,13]. In spite of the efforts have been made to improve the treatment of NSCLC, treatment outcomes of those patients are usually poor. Changes in blood substances are usually observed during the development of various human diseases, and monitoring the changes of those substances in blood may provide guidance for the diagnosis and prognosis of human diseases [14, 15]. In this study, serum levels of GASL1 were found to be significantly lower in NSCLC patients than in healthy controls. ROC curve analysis showed that serum GASL1 can be used to effectively distinguish NSCLC patients from healthy controls. Survival curve comparison showed that the overall survival conditions of patients with low serum level of GASL1 were worse than those with high serum level of GASL1. Those findings indicate that serum GASL1 may serve as a potential diagnostic and prognostic marker for NSCLC. Our data also showed that serum levels of GASL1 are not affected by patients' age, gender, as well as smoking and drinking habits, which have been proved to affect the expression pattern of some other lncRNAs[16-18], indicating the high reliability of GASL1 in the diagnosis and prognosis of NSCLC. We showed that serum levels of GASL1 are not affected by distant tumor metastasis, indicating that serum GASL1 is not a promising biomarker for the prediction of tumor metastasis in NSCLC. In contrast, significant correlations were found between serum levels of GASL1 and tumor size, indicating the involvement of GASL1 in tumor growth of NSCLC. It has been reported[19-21] that TGF-β1 expression promotes tumor metastasis, but inhibits tumor growth in lung cancer[9]. In this study, GASL1 overexpression inhibited NSCLC proliferation and upregulated TGF-β1 expression. In contrast, GASL1 knockdown promoted NSCLC proliferation and downregulated TGF-β1 expression. In addition, TGF-β1 treatment reduced the enhancing effects of GASL1 knockdown on NSCLC cell proliferation. Those data suggest that GASL1 may inhibit cancer cell proliferation by upregulating TGF-β1. It is also worth to note that GASL1 overexpression and knockdown showed no significant effects on proliferation of cells of normal human lung tissue cell line WI-38, suggesting that GASL1 may serve as a potential therapeutic target for NSCLC.

Conclusions

We found that lncRNA GASL1 expression was significantly downregulated in NSCLC. Serum GASL1 is a sensitive biomarker for NSCLC, and low expression level of GASL1 indicated short postoperative survival. GASL1 overexpression upregulated, and GASL1 knockdown downregulated TGF-β1 expression in NSCLC cells, but not in normal lung cells. GASL1 overexpression inhibited and knockdown promoted cancer cell proliferation. Therefore, we concluded that lncRNA GASL1 may inhibit tumor growth of NSCLC by downregulating TGF-β1.

Conflict of Interest

The Authors declare that they have no conflict of interest.

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